BIOCHEMICAL STUDIES OF THE PITCHER LIQUOR OF NEPENTHES.

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(*Read April 14, 1917.*)

As the pitcher of *Nepenthes* gradually develops, a liquor is secreted and occupies the lower portion of its cavity. After the operculum, or lid, has opened, insects are attracted by the nectar which is secreted by glands. The nectar glands are found on the outer surface of the pitcher, more abundantly on the inner surface of the lid, and on the inner edge of the corrugated rim that surrounds the margin of the pitcher. The insects, thus attracted, are tempted down into the pitcher, and pass to a richly glandular zone with a smooth surface—the so-called detentive surface—on which they lose their footing, and are precipitated into the liquor. The insects are then digested by the liquor.

Two theories exist as to the manner in which digestion occurs; each theory is supported by experimental evidence. Hooker,[1] Tait,[2] von Gorup and Will,[3] Vines,[4] Goebel,[5] Clautriau,[6] and Fenner[7] concluded from their researches that the digestion is due to an enzyme

[1] Hooker, *Nature,* 1874, X., 366–372. Report of the Forty-fourth Meeting of the British Association for the Advancement of Science, 1874; Notes and Abstracts of Miscellaneous Communications to the Sections, 1875, pp. 102–116.

[2] Tait, *Nature,* 1875, XII., 251–252.

[3] von Gorup and Will, *Sitzungsberichte der physikalisch-medicinischen Societät zu Erlangen,* 1875–6, VIII., 152–158. *Ber. der deut. chem. Gesell.,* 1876, IX., 673–678.

[4] Vines, *Jl. of Linnean Society, Botany,* 1877, XV., 427–431. *Annals of Botany,* 1897, XI., 563–584; 1898, XII., 545–555; 1901, XV., 563–573.

[5] Goebel, *Pflanzenbiologische Schilderungen,* 1893, II., 186–193.

[6] Clautriau, *Mémoirs couronnés et autres mémoires,* publiés par l'Académie Royale des Sciences, des Lettres et des Beaux Arts de Belgique, Collection in 8°, 1899–1900, LIX., third memoir, 56 pages.

[7] Fenner, *Flora oder allgemeine botanische Zeitung,* 1904, XCIII., 335–434 (especially pp. 358–363).

present in the pitcher liquor. On the other hand, Dubois[8] and Tischutkin[9] concluded from their experiments that the digestion is due to the activity of microörganisms. A third factor to be considered is the autolysis produced by the tissue enzymes of the captured insects.

In the present research, the proteolytic enzyme of the pitcher liquor and the bacteria, which occur in opened pitchers, have been studied separately. The following species and hybrids of *Nepenthes* supplied material for the research: *ampullaria, atrosanguinia, Chelsonii, Claytonii, Dominii, Dyeriana, gracilis, Hamiltoniana, Henryana, Hookeriana, Mastersiana, mixta, Morganiana, paradisæ, Rafflesiana pallida, rufescens, splendida, Wittei.* The plants were grown in the Nepenthes House of the University of Pennsylvania.

Protease of the Pitcher Liquor.

In the study of the protease of the pitcher liquor, pitchers were always selected prior to opening. They were closely watched and the mouth of each pitcher was closed with absorbent cotton as soon as the lid opened; the entrance of insects was thereby prevented, and possible contamination of the pitcher liquor by the tissue enzymes of the digested prey was entirely excluded. The digestion experiments with the pitcher liquor were made in vitro in the presence of a bactericide; bacterial action was thereby excluded. The proteolysis, which was observed, was, therefore, due to enzyme action.

Liquor from both non-stimulated and stimulated pitchers was studied. The experiments on liquor from non-stimulated pitchers were carried out as soon as possible after the opening of the pitchers.

When liquor from stimulated pitchers was desired, recourse was had to mechanical stimulation by chemically inert substances. In some experiments, the glands of the inner wall of the pitcher were stroked repeatedly with a camel's hair brush, and the cotton plug was then inserted in the mouth of the pitcher; the liquor was removed for study on the following day. In other experiments, several round solid glass beads, such as are used in fractionating

[8] Dubois, *Comptes rend. de l'Academie des Sciences,* 1890, CXI., 315–317.
[9] Tischutkin, *Botanisches Centralblatt,* 1892, L., 304–305.

columns, were inserted into the newly opened pitcher; the cotton plug was introduced, and the pitcher and its contents were shaken thoroughly at intervals during one or more days, taking care not to wet the cotton and thereby lose liquor; the liquor was finally removed and used in digestion experiments.

The volume of liquor secreted by a single pitcher was always so small that liquor could not be obtained from the same pitcher both before and after stimulation. Since two pitchers rarely matured on the same plant at the same time, it was impossible to make a comparative study of the liquor from both non-stimulated and stimulated pitchers of the same plant. While the differences, due to individual plants, could not be entirely eliminated, the problem was attacked by several methods for the study of proteolysis, and a number of experiments were conducted according to each method; the results obtained by all the methods lead to the same general conclusions.

Either sodium fluoride or trikresol was used as a bactericide in all the experiments reported below. When sodium fluoride was used, sufficient solid fluoride was added to the mixture of pitcher liquor and substrate to render the final concentration of sodium fluoride one per cent. When trikresol was used, a sufficient volume of a two per cent. aqueous solution of trikresol was added to render the final concentration of trikresol 0.2 per cent.—a concentration which was found satisfactory by Graves and Kober[10] in certain of their experiments with proteases. Whenever the mixture of pitcher liquor and substrate was diluted to a definite volume, the trikresol solution was added before the dilution to the final volume was made.

Unless otherwise stated, the temperature of incubation was 37° C.

In each experiment, a blank or control experiment was carried out with pitcher liquor which had previously been boiled, then cooled to the temperature of the room; the control experiment was carried out in exactly the same manner, in all other respects, as the determination proper. The control was always compared with the determination proper; and due allowance was thus made for the

[10] Graves and Kober, *Jl. Am. Chem. Soc.*, 1914, XXXVI., 751–758.

possible action of any thermostable catalyst present in the pitcher liquor, and also for any action of the reagents on each other.

The following reactions for the detection of a protease were used:

1. The formol-titration of Sörensen.
2. The digestion of:
   (*a*) Carmine fibrin.
   (*b*) Edestan.
   (*c*) Protean of castor bean globulin.
   (*d*) Ricin (Jacoby).
3. The cleavage of glycyltryptophane.

Formol-titration.

Composite samples of liquor, obtained from several pitchers, were used, a different sample for each experiment. The indicator was phenolphthalein.

TABLE I.

Formol-Titration after Digestion.

Liquor from	Substrate.	Weight of Substrate, Gram.	Volume of Pitcher Liquor, C.c.	Total Volume of Digestion Mixture, C.c.	Period of Incubation, Days.	Formol-titration after Deduction of Blank,
Non-stimulated pitchers	Ovalbumen....	0.05	10.0	10.0	4	0.00 c.c. 0.1 *N* NaOH
	Nährstoff Heyden*	0.25	12.5	37.5	4	0.00 c.c. 0.1 *N* NaOH
	Witte peptone..	0.25	12.5	37.5	4	0.00 c.c. 0.1 *N* NaOH
Stimulated pitchers.	Ovalbumen....	0.05	15.0	15.0	3	0.15 c.c. 0.05 *N* NaOH
	Fibrin.....	0.05	15.0	15.0	14	0.45 c.c. 0.1 *N* NaOH
	Ovomucoid....	0.05	5.5	15.5	6	0.10 c.c. 0.1 *N* NaOH
	Nährstoff Heyden*	0.15	15.0	30.0	3	0.20 c.c. 0.1 *N* NaOH
	Witte peptone..	0.25	25.0	50.0	4	2.75 c.c. 0.1 *N* NaOH

* According to Gotschlich (Kolle und Wassermann, Handbuch der pathogenen Mikroorganismen, 2 Auflage, 1912, I., 102), Nährstoff Heyden is a mixture of different albumoses.

In the first series of experiments, reported in detail in Table I.. the pitcher liquor was permitted to act on a given substrate for the time stated. Undissolved protein was then removed by filtration, and was washed on the filter; the combined filtrate and washings were made neutral in reaction. Any precipitated metaprotein was separated by filtration, and was washed on the filter; the combined filtrate and washings were neutralized, if necessary. One half their volume of a neutral, 40 per cent. formaldehyde solution was then added, and the carboxyl groups of the amino acids were immediately titrated with standard sodium hydroxide solution. It should be noted that no acid was added in the digestion experiments of Table I.

A second series of experiments was carried out with edestan as the substrate; in these experiments, the hydrochloric acid, which was used to convert edestin into edestan, was present in the solution during the digestion. The details are given in Table II.

TABLE II.

FORMOL-TITRATION AFTER DIGESTION OF EDESTAN.

Liquor from	Volume of Pitcher Liquor, C.c.	Weight of Edestin, Gram.	Volume of 0.1 *N* Hydrochloric Acid, C.c.	Volume of Water, C.c.	Total Volume of Digestion Mixture, C.c.	Period of Incubation, Days.	Formol Titration after Deduction of blank, C.c. 0.1 *N* NaOH.
Non-stimulated pitchers.....	11.5	0.100	15.0	23.5	50.0	28	0.00
	8.0	0.036	5.4	11.6	25.0	21	0.00
Stimulated pitchers.....	8.0	0.050	7.5	17.5	33.0	25	0.90

In both series of experiments the liquor from *stimulated* pitchers invariably digested the substrate, liberating compounds which were of the nature of amino acids, and responded to the formol-titration. The liquor from *non-stimulated* pitchers did not digest the substrate with the production of such compounds, for the formol-titration invariably was zero.

DIGESTION OF CARMINE FIBRIN.

Two sets of experiments were made using carmine fibrin as the substrate. In both series, the temperature of incubation was that of the room. The liquor from a separate pitcher was used in each

experiment, save in those indicated by an asterisk (*) in the tabulated results. In the latter experiments, composite samples of liquor, obtained from several pitchers, were used.

In the preliminary experiments, the carmine fibrin was swollen in a solution containing 0.2 per cent. hydrochloric acid and 0.2 per cent. trikresol. A definite volume of the resulting jelly was placed in a given volume of pitcher liquor; and sufficient hydrochloric acid (0.6 per cent. solution) and trikresol (2 per cent. solution) were added to produce a concentration of 0.2 per cent. of each of these reagents. The time required for solution of the substrate and other details of each experiment are given in Table III.

TABLE III.

DIGESTION OF GELATINOUS CARMINE FIBRIN BY PITCHER LIQUOR IN THE PRESENCE OF 0.2 PER CENT. HYDROCHLORIC ACID.

Liquor from	Volume of Pitcher Liquor, C.c.	Volume of Carmine Fibrin Jelly, C.c.	Solution of Substrate.	
			Marked in Hours.	Complete in Hours.
Non-stimulated pitchers....	1.50	0.10		13
	*4.75	0.25	31	
Stimulated pitchers.....	1.50	0.10		13
	4.00	0.50	15	24
	4.00	0.50	48	144
	*4.75	0.25		26

In the final series of experiments, 0.2 gram of carmine fibrin was weighed out into a separate tube for each experiment. This series falls into three groups. In group *A*, the carmine fibrin was swollen in its tube in a solution containing 0.2 per cent. hydrochloric acid and 0.2 per cent. trikresol, then was transferred to another tube and immediately used in a digestion experiment. In groups *B* and *C*, unswollen carmine fibrin was used. No hydrochloric acid was used in group *B*; in the other two groups, sufficient 0.6 per cent. hydrochloric acid was added to render the final concentration of that acid 0.2 per cent. Trikresol was used as a bactericide in all three groups, adding sufficient of its 2 per cent. aqueous solution to produce a final concentration of 0.2 per cent. The details of these experiments are recorded in Table IV.

TABLE IV.

TIME REQUIRED FOR DIGESTION OF 0.2 GRAM OF CARMINE FIBRIN BY THE PITCHER LIQUOR.

Group.	Liquor from	Volume in C.c. of Pitcher Liquor.	Time Required for Complete Solution of Substrate.	Reagents Used.
A.	Stimulated pitchers.....	3.5	48 hours	Swollen carmine fibrin and 0.2% hydrochloric acid
		2.5	72 "	
		2.0	93 "	
		3.5	111 "	
		1.0	133 "	
B.	Non-stimulated pitchers..	1.0	Substrate absolutely unattacked after incubation for 70 *days*	Unswollen carmine fibrin; no hydrochloric acid
		1.5		
		2.5		
		3.0		
		3.5		
		5.5		
			Time required for marked digestion of substrate	
C.	Non-stimulated pitchers..	2.5	16 hours	Unswollen carmine fibrin and 0.2% hydrochloric acid
		1.0	52 "	

The results of both series of experiments (Tables III. and IV.) demonstrate that the liquor from both non-stimulated and stimulated pitchers dissolved carmine fibrin in the presence of 0.2 per cent. hydrochloric acid, and that liquor from non-stimulated pitchers had absolutely no digestive action on that substrate in the absence of that acid.

DIGESTION OF EDESTAN.

The solution of edestan was prepared by dissolving 0.1 gram of edestin in 15 c.c. of 0.1 *N* hydrochloric acid, previously diluted with water to a volume of 25 c.c. The details of the various experiments are given in Table V. After incubation of the mixture of pitcher liquor and edestan solution, it was rendered neutral to phenolphthalein by addition of 0.1 *N* sodium hydroxide solution.

In the experiments with liquor from stimulated pitchers, no precipitate formed in the determination proper on neutralization, showing that the digestion of the protean edestan had advanced beyond the metaprotein stage. In the experiments with liquor from non-stimulated pitchers, a precipitate always formed in the

determination proper, but it always was decidedly less voluminous than the precipitate in the corresponding control experiment; therefore digestion of the substrate had occurred, though less rapidly than when liquor from stimulated pitchers was used.

TABLE V.

DIGESTION OF EDESTAN BY THE PITCHER LIQUOR.

Liquor from	Volume of Pitcher Liquor, C.c.	Volume of Edestan Solution, C.c.	Volume of Water, C.c.	Total Volume of Digestion Mixture, C.c.	Period of Incubation, Days.	Precipitate on Neutralization of Experiment Proper After Incubation.
Non-stimulated pitchers...	1	2	2	5	8	Precipitate one half as great as in blank
	4	1	0	5	13	Precipitate one half as great as in blank
Stimulated pitchers.....	20	25	5	50	14	No precipitate
	1	2	2	5	8	No precipitate

DIGESTION OF THE PROTEAN OF CASTOR BEAN GLOBULIN.

A 2 per cent. solution of castor bean globulin in 5 per cent. sodium chloride solution was used. This solution was filtered, if necessary, then mixed with the pitcher liquor; 0.1 *N* hydrochloric acid was next added; a cloudy precipitate of the protean derived from the globulin formed. The presence in the pitcher liquor of a proteolytic enzyme, active in the presence of hydrochloric acid, was shown by the digestion or solution of the protean, the cloudy precipitate gradually becoming less dense, and finally disappearing completely. Liquor from a separate pitcher was used in each experiment; the details are recorded in Table VI. The protean was usually dissolved by the liquor from both non-stimulated and stimulated pitchers.

DIGESTION OF JACOBY'S RICIN.

The reagent was prepared by dissolving 1 gram of Jacoby's ricin and 1.5 grams of sodium chloride in 100 c.c. of water, and filtering, if necessary. The test was carried out by mixing 1 c.c. of pitcher liquor and 3 c.c. of ricin solution, adding 1 c.c. of 0.56 per cent. hydrochloric acid, then incubating. A cloudy precipitate

TABLE VI.

DIGESTION OF THE PROTEAN OF CASTOR BEAN GLOBULIN BY THE PITCHER LIQUOR.

Liquor from	Volume of Pitcher Liquor, C.c.	Volume of Globulin Solution, C.c.	Volume of 0.1 *N* Hydrochloric Acid, C.c.	Volume of Water, C.c.	Total Volume of Digestion Mixture, C.c.	Digestion.
Non-stimulated pitchers...	0.6	2.0	0.5	1.9	5.0	No digestion at end of 14 days
	2.5	2.0	0.5	0.0	5.0	Protean completely dissolved in 14 hours
	1.0	2.0	0.5	1.0	4.5	Protean almost completely dissolved in 29 hours
	1.0	2.0	0.5	1.0	4.5	Protean completely dissolved in 48 hours
Stimulated pitchers.....	2.5	2.0	0.5	0.0	5.0	Digestion of protean marked on 3d day, advanced on 7th, almost complete on 12th day
	0.5	4.0	1.0	4.5	10.0	Digestion of protean marked on 4th day, almost complete on 9th day

of protean, derived from the ricin, separated on the addition of the acid. By the action of a protease, this precipitate was gradually digested or dissolved.

In one experiment, liquor from a *non-stimulated* pitcher was used; the cloudy precipitate was partially dissolved in two days, but had not entirely disappeared at the end of seven days. In another experiment, liquor from a *stimulated* pitcher was used, the cloudy precipitate was markedly digested in two days. Therefore, liquor from both non-stimulated and stimulated pitchers exerted a proteolytic action on the protean.

CLEAVAGE OF GLYCYLTRYPTOPHANE.

Liquor from stimulated pitchers was permitted to act on glycyltryptophane in the presence of toluene as a bactericide; 10 c.c. of pitcher liquor and 2 c.c. of an aqeous solution of the dipeptide (so-called Ferment diagnosticum) were used. In one experiment,

cleavage of the dipeptide had not occurred after digestion for nine days in the incubator. In a second experiment, after digestion for twenty-one days in the incubator, followed by seven days in the room, a distinctly positive reaction for free tryptophane was obtained by the bromine and acetic acid test. Hence, liquor from stimulated pitchers apparently hydrolyzed glycyltryptophane, provided the period of incubation was sufficiently long.

Bacteria of the Pitcher Liquor.

The bacteriological study was made in collaboration with E. Quintard St. John. Unopened and opened pitchers were studied separately.

Unopened Pitchers.—Sterile scissors were used in cutting the plant tissues. The prolonged midrib or tendril, which carries the pitcher, was severed; the top portion of the pitcher was rapidly passed through the flame and was immediately cut off. The cut edge of the pitcher was then flamed rapidly; and the liquor was withdrawn at once with a sterile pipette, and plated on plain nutrient agar. After incubation for four days at 37° C., the plates were examined for bacterial growth.

Twelve pitchers were studied in this manner, the liquor from each pitcher being plated separately. Colonies invariably failed to develop on the plates; hence the liquor in unopened pitchers was sterile.

Opened Pitchers.—Partly opened pitchers, which had not been invaded by insects, were used in two experiments. The liquor from each of these pitchers contained a goodly number of bacteria which grew on plain nutrient agar at 37° C.

All the other experiments were conducted on liquor from open, active pitchers in which insect remains were present.

Total Count.—With each of five pitchers, several successive dilutions of the liquor were sown on plain nutrient agar, and the plates were incubated at 37° C. for four days; the colonies were then counted. The number of bacteria per c.c. of pitcher liquor was as low as 48,000 in one pitcher, and as high as 8,000,000 in another pitcher; the other pitchers gave values: 450,000, 1,200,000, and 1,900,000, respectively.

Several of the colonies were studied separately (*a*) by stained smears, and (*b*) by transfers to lactose bile salt broth. All the microörganisms were rod-like, and a few of them contained spores; none of the transfers developed gas; therefore it may be concluded that members of the family *Bacteriaceæ,* other than the colon-aërogenes group were present.

The liquor in an old pitcher, which was becoming brown at the top, gave a count of 104,000 bacteria per c.c.

Liquefaction of Gelatin.—The liquor from each of two pitchers was sown on nutrient gelatin; the bacteria grew and completely liquefied the gelatin in forty-eight hours.

Tests for the Colon-aërogenes Group.—In two experiments, 1 c.c. of liquor from a single pitcher was sown in lactose bile salt bouillon; on incubation at 37° C., gas developed within seventy-two hours, showing the presence of organisms of this group. A composite sample of liquor, collected from several pitchers, was sown in this medium in several successive dilutions, the greatest dilution being 1 : 10,000; gas developed in even the greatest dilution within seventy-two hours, therefore at least 10,000 organisms of the colon-aërogenes group were present in each c.c. of the liquor.

Certain special media were used in the study of the power of the bacteria to digest proteins, and to decompose simple compounds containing carbon and nitrogen. In the preparation of these media, the directions of Crabill and Reed[11] were followed with modifications. A stock solution of inorganic salts, containing magnesium, ferrous, potassium, chloride, sulphate, and phosphate ions, was prepared as directed by these authors, and was used in the media.

The liquor from each pitcher was studied separately in all the experiments described below. The temperature of incubation was always 37° C.

Production of Tryptophane and Indol from Protein.—Protein, obtained from aleuronat, gave a purple color with glyoxylic acid and sulphuric acid, and therefore contained a tryptophane group. A medium, containing 0.4 gram protein, 20 c.c. 0.1 *N* sodium hydroxide solution, and 80 c.c. of the stock solution of inorganic salts, was prepared and sterilized. One c.c. of pitcher liquor was sown

[11] Crabill and Reed, *Biochem. Bull.,* 1915, IV., 30–44.

in 10 c.c. of the sterile suspension of protein. In one series of eight experiments, indol had not been produced after incubation for ten days. In another series of eight experiments, neither free tryptophane nor indol was present after incubation for twelve days.

Digestion of Proteins by the Bacteria.—The proteins used were: casein, egg albumen, unswollen carmine fibrin, edestin, ricin (Jacoby), protein (prepared from aleuronat). The media were prepared by addition of 2 per cent. of agar and approximately 1 per cent. of *one* of the proteins to the stock solution of inorganic salts, and were then sterilized. The proteins, therefore, served as the sole source of carbon and nitrogen for the bacteria. These media were used in plating experiments, sowing 1 c.c. of pitcher liquor in each plate. Whenever proteolytic bacteria were present in the pitcher liquor, their colonies gradually digested and dissolved the suspended particles of protein over which they grew. The plates were examined at intervals until drying of the media rendered further observation useless. The bacteria grew and colonies developed on the vast majority of the plates, as may be seen by reference to Table VII., which gives certain details of these experiments.

TABLE VII.

DIGESTION OF PROTEIN MEDIA BY BACTERIA, PRESENT IN LIQUOR OF OPEN PITCHERS.

Protein Used.	Number of Experiments Plated.	Number of Experiments Showing Growth.	Growth Occurred in Days.	Digestion Begun Between Days.	Total Period of Incubation Days.
Casein	8	7	3	3 to 5	12
Egg albumen	15	11	3 to 9		12 to 14
Carmine fibrin	6	6	3	5 to 9	9
Edestin	4	4	3	3 to 9	12
Ricin	3	2	3 to 5	By 9th day	12
Protein (from aleuronat)	8	8	3	3 to 5	12

The suspended egg albumen was not even partially digested; possibly this was due to the presence of non-coagulable ovomucoid, and its utilization by the bacteria as a source of carbon and nitrogen. The other suspended proteins were gradually digested, the digestion becoming more marked as the period of incubation increased; however, complete digestion and disappearance of the suspended particles had never occurred by the end of this period.

Sufficient liquor was not always obtained from a single pitcher to permit plating on all six media. However, a general tendency was noted that, when the microörganisms present in the pitcher liquor grew on one of these media, they grew on all the media, and usually exerted a visible digestive action on all the proteins.

Production of Basic Compounds.—A study was also made of the formation of basic compounds by the action of the microorganisms on simple organic compounds, which served as the sole source of carbon and nitrogen in the medium. The solid media were prepared by addition to the stock solution of inorganic salts of 2 per cent. of agar, 0.5 per cent. by volume of a 2 per cent. solution of rosolic acid in 60 per cent. alcohol, and *one* of the following compounds: glycocoll (an amino acid), acetamide (an acid amide), asparagin (which is both an amino acid and an acid amide), ammonium lactate (an ammonium salt of an organic acid). One per cent. of asparagin was used, the other compounds in molecular concentration equal to that of the asparagin. These media were always sterilized by the discontinuous method. Plating experiments were made, sowing 1 c.c. of pitcher liquor in each plate. The production of basic compounds by microörganisms growing on these media was indicated by a red color of the medium beneath and surrounding the colony. Sterile plates were always poured as controls, to be used in determining the changes in color of the experiments proper.

The number of experiments plated on each of these media was:

Glycocoll rosolic acid agar..... 7 experiments.
Acetamide rosolic acid agar..... 7 experiments.
Asparagin rosolic acid agar.....22 experiments.
Ammonium lactate rosolic acid agar..... 7 experiments.

The period of incubation was from ten days to a fortnight, the plates being inspected at intervals until further observation was rendered useless by drying of the media. Colonies developed in all save one of the experiments, the sole exception being a plate of glycocoll rosolic acid agar. The colonies were usually apparent by the third day; though, in a few instances, they grew so slowly that they became apparent only at the tenth day. The organisms always

produced an alkaline reaction, *i. e.,* a reddening of the medium. This red coloration gradually spread from the colonies as centers over the entire plate, and was quite marked, as a general rule, by the third or fifth day. In those experiments in which colonies developed but slowly, the red coloration was noted by the tenth day. Hence the microörganisms produced basic compounds from the substrates. During the last portion of the period of observation, from the tenth to the fourteenth day of incubation, ofttimes the medium changed in reaction and became acid to rosolic acid, nevertheless the colonies themselves remained alkaline.

Included in the above experiments was a group in which the liquor from each of seven pitchers was plated on all four rosolic acid agars—glycocoll, acetamide, asparagin, and ammonium lactate. The bacteria grew and produced an alkaline reaction at about the same rate in all four media. In these experiments, a record was also kept of the odor of the cultures; quite frequently the plates were characterized on the third day by an odor recalling that of ammonia or amines; this odor was rarely present on the tenth day.

Hence the microörganisms were able to utilize glycocoll, acetamide, asparagin, and ammonium lactate, which formed their sole source of carbon and nitrogen, and were able to produce basic nitrogenous compounds from these substrates.

General Summary.

The following conclusions are based on the chemical studies:

Using the *formol-titration,* it was found that liquor from non-stimulated pitchers lacked proteolytic power, while liquor from stimulated pitchers produced proteolysis of a number of substrates: ovalbumen, fibrin, ovomucoid, Nährstoff Heyden, and Witte peptone. In the presence of very dilute hydrochloric acid, edestan was digested by liquor from stimulated pitchers, but not by that from non-stimulated pitchers.

Carmine fibrin was not dissolved by liquor from non-stimulated pitchers in the absence of acid, but was digested and dissolved by the liquor from both non-stimulated and stimulated pitchers when 0.2 per cent. of hydrochloric acid was present in the reaction mixture.

Edestan was digested by the pitcher liquor in the presence of very dilute hydrochloric acid; liquor from stimulated pitchers produced a more rapid digestion than did liquor from non-stimulated pitchers.

Liquor from both non-stimulated and stimulated pitchers usually dissolved the *protean of castor bean globulin* in the presence of very dilute hydrochloric acid.

Jacoby's *ricin* was dissolved by liquor from both non-stimulated and stimulated pitchers in the presence of very dilute hydrochloric acid.

Liquor from stimulated pitchers apparently produced cleavage of *glycyltryptophane,* when the period of incubation was sufficiently long.

Liquor from *non-stimulated* pitchers exerted proteolytic action only in the presence of acid, failing to produce proteolysis in the absence of acid.

Liquor from *stimulated* pitchers exerted proteolytic action in both the presence and the absence of acid.

The manner in which stimulation causes the pitcher liquor to acquire *active* proteolytic power is a field for further research. Stimulation may produce a change in the hydrogen ion concentration[12] and thus render the reaction favorable for the activity of a protease already present in the pitcher liquor; or it may cause the activation of a zymogen already present; or it may give rise to an increased secretion of protease by the glands of the pitcher.

In the presence of acid, certain substrates—especially edestan—were digested by liquor from stimulated pitchers more rapidly than by liquor from non-stimulated pitchers.

The following conclusions are drawn from the bacteriological studies:

Liquor taken aseptically from unopened pitchers was sterile.

[12] Since this paper was presented, an abstract of a monograph by Jenny Hempel entitled "Bidrag til Kundskaben om Succulenternes Fysiologi" (Copenhagen, H. Hagerup, 1916, 147 pp.) has appeared in *Physiological Abstracts,* 1917, II., 146 (issued in May, 1917). The following quotation is taken from this abstract. "The sap of the stimulated pitcher of *Nepenthes* gives values for the hydrogen ion concentration greater than 10^{-7}, but unstimulated pitchers give no definite value."

A goodly number of bacteria were present in the liquor of pitchers which had partly opened, but had not yet been invaded by insects.

The bacterial content of the liquor of open active pitchers, which contained insect remains, was quite high—from 48,000 to 8,000,000 per c.c. of liquor; the organisms were rods. These bacteria liquefied gelatin, and formed colonies on solid media (agar) in which the sole source of carbon and nitrogen was either a protein or a simple organic compound. They usually digested the protein (casein, egg albumen, carmine fibrin, edestin, Jacoby's ricin, protein from aleuronat), but at an exceedingly slow rate. They decomposed the simple nitrogenous organic compounds (glycocoll, acetamide, asparagin, ammonium lactate), frequently producing an odor like that of ammonia and amines, and always imparting an alkaline reaction to the medium; this reaction subsequently changed to acid in some experiments, but the colonies always remained alkaline. The bacteria did not produce either tryptophane or indol from aleuronat protein, when sown on a medium in which the latter was the sole source of carbon and nitrogen. Organisms of the colon-aërogenes group were present; on the average, each c.c. of pitcher liquor contained 10,000 organisms of this group.

The chemical and bacteriological studies taken together lead to these conclusions.

The protease of the pitcher liquor is the chief factor in the digestion of the insects in the pitcher. The bacteria, which occur in the liquor of opened pitchers, play merely a secondary part, as is shown by the slowness with which they digested proteins.

The bacteria and the *Nepenthes* plant live in symbiosis; the bacteria obtain their food from the digested insects and assist, to a limited extent, in the digestion of the insects.

The tissue enzymes of the insects, of course, may assist in the digestion by causing the insects' tissues to undergo autolysis.

Note on the Biochemistry of the Pitcher Liquor of Sarracenia.

Closely related to the family *Nepenthaceæ,* with its single genus *Nepenthes,* is the family *Sarraceniaceæ,* consisting of three

genera: *Sarracenia, Darlingtonia,* and *Heliamphora.*[13] The following experiments were made on the pitchers of two species of *Sarracenia—flava* and *minor*—by Frank M. Jones, E. Quintard St. John, and the author, and are mentioned in this place, since they indicate that the digestive action in the pitchers of *Sarracenia* is likewise due to a proteolytic enzyme.

Liquor was obtained from unopened pitchers of *Sarracenia flava,* growing in their native habitat, and was used in test-tube experiments. The liquor digested edestan in the presence of very dilute (less than 0.1 per cent.) hydrochloric acid, and rapidly digested carmine fibrin—swollen or unswollen—in the presence of 0.2 per cent. hydrochloric acid, 0.2 per cent. of trikresol being used as a bactericide. Liquor was also obtained from open pitchers; it had been diluted by rain water, but rapidly digested carmine fibrin in the presence of hydrochloric acid and trikresol, the concentration of these reagents being that just stated.

By means of culture experiments, it was determined that the contents of unopened pitchers of *Sarracenia flava* and *Sarracenia minor* (*Sarracenia variolaris*) were bacteriologically sterile.

The contents of opened pitchers of these species, which contained insect remains, were also studied bacteriologically. The test for the liquefaction of gelatin was conducted as directed by Rivas;[14] the medium was liquefied; and both motile and non-motile rod-like microörganisms were recovered from the resulting cultures. The contents of these pitchers were also plated on certain of the special agar media described in the preceding pages. The bacteria grew on casein agar and on protein (from aleuronat) agar, and digested these proteins, but at an exceedingly slow rate. The bacteria also grew on the various rosolic acid agars—glycocoll, acetamide, asparagin, and ammonium lactate—changing the reaction of the medium to alkaline, and producing an odor of ammonia or of bases; on prolonged incubation the reaction changed to acid. Organisms of the colon-aërogenes group were found to be present by their reaction with lactose bile salt bouillon.

[13] Macfarlane, Engler's Pflanzenreich, 1908; IV., 110, Sarraceniaceæ, 34 Heft; and IV., 111, Nepenthaceæ, 36 Heft.

[14] Rivas, *Jl. Am. Med. Assoc.,* 1908, L., 1492–1495.

These experiments indicate that in *Sarracenia,* as in *Nepenthes,* the protease of the pitcher liquor plays the leading rôle in the digestion of the captured insects. The bacteria of the pitcher liquor have merely a minor rôle; they live in symbiosis with the *Sarracenia* plant, draw their food from the digested insects, and assist to a limited degree in the digestion of these insects. Tissue enzymes of the insects doubtless also participate in the process of digestion.

The author desires to record his deep indebtedness to Dr. John M. MacFarlane, director of the botanic garden and laboratory of this university, for the material and facilities placed at his disposal for the prosecution of this research, during 1914–1916.

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